Title: Analgesic Effect of Bumetanide on Neuropathic Pain in Patients with Spinal Cord Injury

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Abstract

Objectives: The current study evaluated the analgesic effects of bumetanide as an adjunctive treatment in the management of neuropathic pain following spinal cord injury. The peripheral expression of Na-K-Cl-cotransporter-1 (NKCC1) and K-Cl-cotransporter-2 (KCC2) genes in polymorphonuclear lymphocytes (PMLs) assessed as a possible biomarker indicating central mechanisms underlying the observed response.

Methods: Through an open-label, single arm, pilot trial of bumetanide (2mg/day), as add-on treatment conducted in 14 SCI patients for 19 weeks. This study consisted of 3 phases: pre-treatment (1 month), titration (3 weeks), and active treatment (4 months). Ultimately, 9 patients completed the study. The primary outcome variables were the endpoint pain score using the numeric rating scale (NRS), and also the short-form McGill Pain Questionnaire. Secondary endpoints included the Short-Form Health Survey that assesses the quality of life. Blood samples were collected and used for determining the expression of NKCC1 and KCC2 genes in transcription and translation levels.

Results: Bumetanide treatment significantly decreased average pain intensity according to the NRS and the short form of the McGill Pain Questionnaire scores. Baseline expression of KCC2 protein was low between groups and increased significantly following treatment (P<0.05). In the current study, pain improvement accompanied by the greater mean change from the baseline (improvement) for the overall quality of life.

Conclusions: These data highlighted the analgesic effect of bumetanide on neuropathic pain and indicated the probable role of upregulation of KCC2 protein and involvement of GABAergic disinhibition in producing neuropathic pain.

Keywords: Bumetanide, NKCC1, KCC2, Neuropathic Pain, Spinal Cord Injury, GABA
Introduction

Spinal cord comprises the most critical neural pathways for multidirectional transportation of various sensations and commands in a precisely computed and condense framework (Arle, Iftimia, Shils, Mei, & Carlson, 2019; Ben-Ari, 2017); such that even small-sized perturbations could emerge into a broad unpredictable array of pathophysiological events within a temporally-inconsistent scope (Keller, Beggs, Salter, & De Koninck, 2007). One prominent sequels of these CNS-confined injuries is a class of pain which is called the central neuropathic pain that frequently leads to the divergent neurological manifestations (burning, stabbing and pain that is similar in quality to electric shock)(Watson & Sandroni, 2016) and neuropsychological complications (anxiety, depression, difficulties with attention, concentration, memory, problem solving and abstract reasoning)(Murray et al., 2007). Spinal cord injury (SCI) is an injury to the spinal cord which is associated varying degrees of sensory and/or motor deficits and paralysis (Kraus, Franti, Riggins, Richards, & Borhani, 1975). In comparison to sexual disabilities and movement limitations, this category of pains could affect the quality of life and community participation more severely (Vranken, 2013).

Loss of inhibitory neuronal cells or inappropriate blockade of the current inhibition in the pain suppression hierarchy are the direct subsequent of the injury or indirect response to the remodeling of the structure of the spinal cord transmitting pathways (Zholudeva et al., 2018). SCI-induced disinhibition causes abnormal interactions between the sympathetic and sensory pathways and also facilitates pain information transition to the perception centers of the cerebrum. These conditions lead to hyperexcitability and oversensitivity (Kramer et al., 2017; Vranken, 2013). Gamma-aminobutyric acid (GABA) is playing a vital central role in the pain perception matrix. In
physiological conditions, adult neurons have low intracellular Cl- levels underlying the inhibitory role of GABA (Schulte, Wierenga, & Bruining, 2018). In contrast, in a wide range of pathological conditions including spinal cord lesions, chronic pain, brain trauma, autism and various types of epilepsies, neurons have high level of intra-cellular Cl- which leads to excitatory GABA actions (Gwak & Hulsebosch, 2011). The expression ratio of two electroneutral co-transporters, Na-K-Cl-cotransporter-1 (NKCC1) that imports chloride ions into the cells and export potassium and sodium out of the many cell types and K-Cl-cotransporter-2 which exports chloride out of the cells, has been a determining factor in shaping the GABA polarity (Ben-Ari, 2017). The high concentration of chloride due to up-regulation of NKCC1 or down-regulation of KCC2 makes the GABA an excitatory and triggering agent for neuropathic pain (Hasbargen et al., 2010; Mòdol, Cobianchi, & Navarro, 2014). Peripheral nerve injury-induced increases in NKCC1 activity within dorsal root ganglia, ventroposterior thalamic nucleus, and the primary sensory cortical regions has been evident to be playing a role in the allodynia and hyperalgesia (Mòdol et al., 2015). Drugs which target these transporters and restore GABAergic inhibitory responses might be a novel therapeutic strategy for alleviating the neuropathic pain. Bumetanide is a sulfonamide-derived diuretic that as a specific NKCC1 inhibitor might have an analgesic effect. Intra-thecal injection of bumetanide has increased withdrawal latency tension, reduced thermal hyperalgesia and attenuated allodynia and hyperalgesia in pain characteristics induced by paclitaxel in one rat model (He, Xu, Huang, & Gong, 2014). Another study showed that bumetanide could attenuate mechanical allodynia in SCI model in rats (Chen et al., 2014).

Considering potentials of bumetanide in restoring central and peripheral inhibitory arm of balance and also the refractoriness of neuropathic pain following spinal cord injury (around 30% of the patients report a 50% decline in their pain symptoms) (Siddall & Loeser, 2001) to the current
pharmacotherapeutic approaches, the central hypothesis of the present investigation is whether this NKCC1 blocker might attenuate pain associated scores and improve quality of life.

Material and Methods

Study design
This study conducted as an open-label, single arm, pilot trial of bumetanide in an add-on protocol for SCI patients that confirmed to have neuropathic pain diagnosis. The study protocol approved by the ethical committee of Tehran University of Medical Sciences and registered in Iranian Registry of Clinical Trials (IRCT201407155368N2). All patients signed written informed consent. Referred patients to the SCI center of Imam Khomeini Hospital were evaluated based on eligibility criteria. Neuropathic pain diagnosis made according to the International Association for the Study of Pain (IASP) Special Interest Group on Neuropathic Pain (NeuPSIG) in 2008 (Finnerup et al., 2016). Participants asked to rate their neuropathic pain symptoms based on the Short-Form McGill Pain Questionnaire 2 (SF-MPQ-2) (Kachooei et al., 2015) and pain intensity based on modified the numerical rating scale (m-NRS). Moreover, the Iranian Short-Form health survey (SF-36) employed to evaluate patients’ quality of life (QOL) state.

All participants in the study were men aged 32-50 years old and diagnosed with pain following SCI by a contributing specialist. Included patients had been experiencing moderate intensity pain for over 3 months (NRS ≥ 4). Exclusion criteria for patients were age over 50 years, advanced liver, kidney and, heart disease. Patients who participated in other clinical trials in the previous three months were also excluded. Having significant neurological or psychological disorder
unrelated to NP that can modulate pain perception were the other exclusion factors. Patients monitored for stabilizing analgesic dose for a month before adding the bumetanide.

**Healthy Control:**

Healthy subjects were assessed according to a checklist for the general medical state particularly head traumas, seizures, and neuropsychological disorders. The selected ones did not report any medication, alcohol or substance use. Both groups were matched in age and education scores.

Venous blood samples (10ml) collected from patients and ten age and sex-matched healthy controls for peripheral blood mononuclear cells (PBMCs) isolation.

**Treatment Protocol and Outcome Variables**

After the baseline assessment, bumetanide tablets administered orally with an initial dose of 0.5 mg/day and increased 0.5 mg every week. After 3 weeks of drug titration, a minimum target dose of 2 mg/day (1mg twice daily) achieved. At the baseline and after the titration period, outcome variables and drug safety assessed at a monthly visit.

The primary outcome measured as change in mean pain score compared to the baseline on an m-NRS scale. The m-NRS score indicates pain intensity ranging from 0 “no pain” to 10 “the worst pain imaginable”. The I-SF-MPQ-2 (Kachooei et al., 2015) comprises of 4 parts, including continuous (throbbing pain, cramping pain, gnawing pain, aching pain, heavy pain, tender), intermittent (shooting pain, stabbing pain, sharp pain, splitting pain, electric-shock pain, piercing), neuropathic (hot-burning pain, cold-freezing pain, pain caused by light touch, itching, tingling or “pins and needles”, numbness), and affective (tiring-exhausting, sickening, fearful, punishing-cruel) subscales; the items that were considered as primary outcome measurements. The total pain
score calculated by the average score in questions. Moreover, the SF-36 health survey that validated in the Iranian assessed as secondary outcome measurements for QOL was applied. It measures eight health-related concepts: physical functioning (10 items), role limitations due to physical problems (4 items), bodily pain (2 items), general health perceptions (5 items), vitality (4 items), social functioning (2 items), role limitations due to emotional problems (3 items), and perceived mental health (5 items). Each scale score is rated from 0 to 100, with a higher number indicating better health status.

**Classification of Evidence**

This study provides Class IV evidence indicating that orally administered 2mg daily bumetanide could be a safe and effective medical option during 4-months treatment in participants with neuropathic pain following spinal cord injury.

**Quantitative Real-time RT-PCR**

Peripheral blood mononuclear cells (PBMC) isolated from 5 ml venous blood sample which drawn into EDTA-containing tube and diluted [1:1] with phosphate-buffered saline (PBS)]. Then layered on top of Ficoll solution (Ficoll-GE Healthcare, Sigma Aldrich) centrifuged at 3000 RPM in 18°C. Afterward, the monocyte layer collected, washed in PBS twice and centrifuged for 5 minutes at 3000 RPM.

Total RNA extracted from the cells using the TriPure Isolation Reagent Isolation Kit (Roche, Germany) according to the manufacturer’s instructions. 1µg of each RNA sample used for cDNA synthesis in a 5-minute at 85°C and 15 minutes’ reaction at 37°C using the reverse transcriptase (Takara, Japan) in the presence of random hexamer, oligo dt, and RNase inhibitor. The regulation
of selected genes was accredited by the quantitative RT-PCR, which was performed in 10 μl reaction volumes using SYBRR-Green PCR Master Mix, including 0.5 μl reversed-transcribed cDNA, 4μl RNase free water, 5 μl 5x primer script plus SYBR & Fluorescein (Takara, Japan), and 0.5 μl primers (10 pmol/μl).

Polymerase chain reaction (PCR) analysis was conducted using the qRT-PCR detection system (Applied Biosystem, one step, RT- RCP Germany) in special 48-well plates under the following conditions: 1 min at 95 °C, 40 cycles of the 30s at 95°C and 1 min at 60°C. All qRT-PCR steps performed in triplicate. PCR products fractionated by 2% agarose gel electrophoresis and the bands visualized by ethidium bromide and photographed with a UVP Imaging System (UVP Company, USA). Hypoxanthine phosphoribosyltransferase1 (HPRT1) served as an internal control and control group samples were used as the reference sample as proliferation markers. The primers were as follows: KCC2, 5′- GTTTCTTCCTGATGTCGA-3′ and 5′-CATAATACCAGGACGA-3′; NKCC1, 5′- TGACTTGAGAGAAGGTGAC-3′ and 5′-TGTTTGGCTTCATACGAC-3′; and housekeeping HPRT1, 5′-CCTGGCGTCGTGATTAGTGA-3′ and 5′- AAGACGTTTCAGCCTGTCCAT-3′. Outcomes were calculated according to the ΔΔCT method.

**Western Blots**

Peripheral monoclonal cells homogenized in lysis buffer (pH 7.4). The extracted proteins quantified using a Bradford test. For Western blot analysis, 40 μg of each protein sample separated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 10% gel. After electrophoresis, proteins transferred to the polyvinylidene difluoride membranes. Blots blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% tween 20 (TBS-T) for 2 hours at room temperature.
Membranes incubated overnight at 4°C with rabbit polyclonal antibody NKCC1 (1:500) (Abcam) and KCC2 (1:1100) (Abcam) and β-actin anti-rabbit polyclonal antibody (1:3000) (Abcam). Following 15 minutes’ washes with TBS-T buffer for three times, membranes incubated for 60 minutes with secondary antibodies at room temperature (1:5000) (Abcam). Immunoreactivity revealed by ECL (Amersham Biosciences, Freiburg, Germany). Quantitative analysis carried out by the monomeric band data with the Image J software. In order to analysis, background density subtracted from the NKCC1 and KCC2 receptor band density and normalized to β-actin, which used as the loading control.

**Statistics**

Statistical package for the social sciences (SPSS Version 25.0) and Graph Pad Prism 7 used for data analysis. The efficacy analyses performed by the intention-to-treat (ITT) method. ITT population defined as patients who took at least one dose of bumetanide and provided at least one baseline and one post-baseline efficacy assessment. The efficacy endpoint was the change in the mean monthly pain score on an m-NRS and pain items of the I-SF-MPQ-2. Values expressed as means ± standard deviation (SD). The endpoints compared using the linear mixed model which incorporates all the assessments at baseline, and every month during the study.

Non-parametric tests (Mann–Whitney U test, Wilcoxon matched-pairs signed rank test) employed for independent and dependent t-test analysis. P-values less than 0.05 considered as significant. Pearson’s correlation coefficient calculated for the correlation between the mean change from baseline of 4-month pain scores, QOL assessment, NKCC1 and KCC2 expression change.
Results

Clinical outcomes

Efficacy Assessment

Over a four-month experiment period, 14 patients included in the study, 3 patients withdrew from the protocol during the first week of the titration phase, because of the adverse effects. The ITT population for the efficacy analysis comprised 11 patients receiving bumetanide up to the second month of treatment and 9 patients completed 4 months’ duration of the study. The baseline demographic and clinical data summarized in Table 1.

Mixed model analyzing showed that neuropathic pain associated intensity score decreased significantly \((P < 0.001); \text{Table 2}\). There were no statistical differences in continuous, intermittent, and affective parts of I-SF-MPQ-2, but total reduced \((P < 0.002); \text{Table 2}\). Moreover, NRS decreased significantly between the two phases of the trial \((P < 0.04); \text{Table 2}\).

The SF-36 completed by the patients before and after bumetanide treatment. Our data demonstrated the greatest change from baseline (improvement) in total SF-36 score \((P = 0.03)\), general health \((P = 0.03)\), mental health \((P = 0.001)\), role emotional \((P = 0.002)\), and bodily pain \((P = 0.001)\) as SF-36 subscales.

Safety Assessment

Drug safety evaluated based on the category and frequency of the adverse effects reported by the patients. Blood samples were taken and assessed at each visit for chemistry analysis, liver function tests (LFTs), as well as the urine samples (lab data presented in table 1).
Five patients withdrew from the study with personal consent; three patients during the titration period, and two patients at the end of the second month of study. All of the dropouts were principally due to the adverse effects such as intolerance of diuretic effect and the difficulty during evacuation and incontinence. Periodic electrolyte concentration evaluation did not indicate any significant changes during the course of the bumetanide treatment.

**NKCC1 and KCC2 mRNAs Expression Levels**

Baseline comparison analysis of the expression levels of KCC2 (P=0.04) and NKCC1 (P=0.01) showed significant difference between healthy controls and patients (figure 1). Afterward, we evaluated post-treatment expression of KCC2 and NKCC1 in a two-month interval (figure 2). The transcription level of these genes changed significantly following treatment (KCC2: P=0.04, NKCC1: P=0.001).

**NKCC1 and KCC2 Proteins Expression Levels**

Western analysis showed the significant difference between protein levels of NKCC1 in patients compared to healthy controls. However, we observed a statistically remarkable low expression level of KCC2 in patients compared to healthy controls (P=0.004) (figure 3). Post-treatment evaluation of NKCC1 and KCC2 proteins indicated meaningful increased KCC2 level (P=0.02), and decreased NKCC1 level (P=0.007) protein was significantly changed after treatment (figure 3).

**Correlation Analyses between Variables**

Pearson’s correlation coefficients between SF-36 total score change from baseline with SF-36 subscale scores, NP, SF-McGill-Q total score, and KCC2 that changed significantly in the study, presented in Table 4. The correlation coefficients were 0.68 for NRS (P= 0.021), 0.72 for mental health (P= 0.01), and 0.82
for bodily pain (P = 0.002) (Table 4). In the current analyses, more pain improvement generally showed greater mean change from baseline (improvement) for overall quality of life.

**Discussion**

Patients who included in the present study have been suffering from neuropathic pain after SCI. Our results showed that treatment with bumetanide for 4 months in patients with SCI, causes significant reduction in pain intensity based on NRS. In addition, spinal cord injury causes adverse sequels on various aspects of people's lives that correlate with lower quality of life (Gurcay, Bal, Eksioglu, & Cakci, 2010). In this study, the SF-36 assessment applied to examine different aspects of quality of life. Our results demonstrated that oral administration of bumetanide can also improve QOL of SCI patients. The present results also provide some considerable information. First, bumetanide tolerated by 9 patients. In fact, the main side effect was polyuria, an expected consequence that was not tolerated by five SCI patients. Second, as an add-on treatment to analgesic drugs, bumetanide relieved some of the NP symptoms. Despite the low concentrations of the bumetanide through the central nervous system following systemic administration (Kaila, Price, Payne, Puskarjov, & Voipio, 2014), recent studies indicated that bumetanide is able to control some aspects of neurological and neuropsychological disorders such as epilepsy (Gharaylou et al., 2019), schizophrenia (Rahmanzadeh et al., 2017), autism (James, Gales, & Gales, 2019) and Parkinson disease (Damier, Hammond, & Ben-Ari, 2016). Previous studies have shown that in animal models of spinal cord injury, the use of bumetanide reduces allostynia and hyperalgesia (Kim et al., 2017; Mòdol et al., 2015; Pitcher, Price, Entrena, & Cervero, 2007).
In the present study, we identified a significant low levels of the KCC2 protein and mRNA expression and high level of NKCC1 in the lymphocytes of the patient group compared with healthy group. As we could not measure the state of the transcription level and proteomic details of the living human brain, and also due to the mirror-like interaction between the CNS and immune system component, we measured the peripheral expression of these transporters. Brain and the immune system use a common biochemical language, where scientists can track changes of the central nervous system in the immune system. There are some reports of the correlation between the state of the mental health and chloride transporters expression in human peripheral blood mononuclear cells (Bhandage et al., 2015) indicating the interaction between the CNS and immune system. We found that compared to healthy subjects, SCI's patients expressed lower levels of KCC2 proteins and mRNA in their peripheral blood. One previous animal study revealed lower KCC2 expression in SCI model during neuropathic pain development (Campbell & Meyer, 2006). We noticed a significant increase in KCC2 protein expression level following bumetanide treatment.

The current results also showed that bumetanide therapy decreased NKCC1 gene expression in SCI patients which is in line with the previous study (Mòdol et al., 2014). Consistent with this study, the results of a study demonstrated that bumetanide phosphorylates NKCC1 and minimizes downregulation of KCC2 and reduces neuropathic pain after peripheral nerve injury (Mòdol et al., 2014). Studies have shown that increased NKCC1 expression and reduced KCC2 expression have a prominent role in neuropathic pain (Hasbargen et al., 2010) who reported a reduction in neuropathic pain after KCC2 recovery (Sánchez-Brualla et al., 2018).

Hypofunction of GABAergic inhibitory tone is an important factor in the enhanced synaptic transmission which leads to neuronal hyperexcitability in dorsal horn neurons following spinal
cord injury (Gwak & Hulsebosch, 2011). Bumetanide as a highly specific NKCC1 chloride importer antagonist efficiently restores low level of intra-cellular Cl- leading to resuming GABAergic inhibitory tone and attenuates many disorders in experimental conditions and in some clinical trials (Ben-Ari, 2017). Certain Studies have demonstrated that blockade of NKCC1 is able to dramatically reduce allodynia and hyperalgesia. Decrease in expression of NKCC1 following the use of bumetanide attenuate many neurological and psychiatric disorders (Ben-Ari, 2017). Moreover, experiments on Down Syndrome mice have shown that the imbalance of NKCC1 and KCC2 expression results in GABAergic stimulatory function that returns to inhibitory GABAergic function after bumetanide treatment (Deidda et al., 2015). In vitro, bumetanide, as a blocker of NKCC1, reverses the GABAergic inhibitory response by decreasing intracellular chloride levels, thereby reducing the severity of the electrical and behavioral manifestations of the GABAergic stimulus response in many pathological conditions (Corradini et al., 2018; Doyon, Vinay, Prescott, & De Koninck, 2016). In this regard, certain previous researches have documented some associated evidence such as a significant increase in KCC2 expression and decline in NKCC1 expression (Price, Cervero, & Koninck, 2005), and degrees of relief in neuropathic pain following administration of bumetanide (Cramer et al., 2008).

Some evidences showed that patients with chronic kidney disease and congestive heart failure after intravenous administration of a high dose of bumetanide (2 mg / h), showed symptoms of neuropathic pain, like light touch tenderness, that after discontinue symptoms resolved (Shisler, Austin, Delpire, Sawyer, & Pandey, 2014). It seems that, these unknown symptoms would be due to adverse effect of high dose of bumetanide (Howard & Dunn, 1997; Shisler et al., 2014). Patients in this study received bumetanide at a dose of 2 mg/ day with 12 hours interval. Regarding to the fact that bumetanide has approximately 500-fold more affinity to NKCC1 than KCC2, so
bumetanide inhibits NKCC1 at low dose without significant effect on KCC2 (Payne, Rivera, Voipio, & Kaila, 2003). It seems that, in our study bumetanide has been implicated in the reduction of neuropathic pain through the NKCC1 blockade. Considering a four-week titration period for each individual, enabled us to detect early possible adverse effects which is especially critical since bumetanide has shown to be pain inducing if not prescribed at the appropriate dose (Shisler et al., 2014).

To our knowledge, these observations provide the first evidence of the efficacy of bumetanide on neuropathic pain and modulation of QOL in SCI patients. Nevertheless, the exact mechanisms by which bumetanide could control chronic neurological symptoms is unclear. It might be assumed that bumetanide reduces the symptoms of pain via resuming of GABA related inhibitory function. Further study using case-control randomized double-blind will elucidate the specificity of this observation.

Our study limitations include the open-label and single-arm study design, involving a relatively small sample size, with the lack of a control group. The gold standard for clinical trials is a well-conducted randomized controlled trial with confirmed blinding throughout the study period. Initially, in the test arm, patients may withdraw due to adverse effects or lack of tolerance to the drug especially diuretic effect of bumetanide, but in the placebo arm, patients may withdraw due to lack of efficacy. Moreover, the diuretic actions of bumetanide effect the blinding procedure. Clearly, this trial view as a source of data on safety. Secondly, this is the first clinical trial of bumetanide in human SCI subjects with neuropathic pain. Bumetanide is a loop diuretic, and its safety not sufficiently assessed in these patients. We need to have sufficient proof to proceed with a large trial and to examine the feasibility of doing such an assessment. We think our study is the first step toward a future fully controlled trials.
In conclusion, the current study pointes towards the potential therapeutic effects of bumetanide on neuropathic pain in patients with spinal cord injury. Bumetanide acts via increase in KCC2 expression and decline in NKCC1 expression. Accordingly, bumetanide as a highly specific NKCC1 chloride importer antagonist, efficiently restores low level of intra-cellular Cl- leading to resuming GABAergic inhibitory tone and attenuates neuropathic pain.

**Data Archiving**

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Statement of Ethics**

The study protocol approved by the ethical committee of Tehran University of Medical Sciences and registered in Iranian Registry of Clinical Trials (IRCT201407155368N2). We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers/animals were followed during the course of this research.

**Authors’ Contributions**

LZ contributed in study design and conceptualization, conducted in the patient follow-up process, drafting and writing of the manuscript.

ZGH mainly contributed in data Analysis, and purification of all RNA and proteins and also in the primary draft of the manuscript.

MH contributed in study conceptualization, interpretation of results and also the revision of the manuscript.
HM was the main responsible for patient recruitment and selection and also in the revision of the manuscript.

FR contributed in the data analysis and result interpretation.

FN was the principal project designer and supervisor, also contributed in the critical revision and submitted approval.
References:


Figure 1: KCC2 and NKCC1 mRNA expression in peripheral blood mononuclear cells between control healthy and spinal cord injury patients were analyzed by quantitative Real-Time PCR. Normalized gene expression levels were given as the ratio between the mean value of the target gene and that for the HPRT1 in patients sample and control. Each column represents mean ± SEM. *P≤ 0.05, **P ≤ 0.01.
Figure 2: KCC2 and NKCC1 mRNA expression in peripheral blood mononuclear cells before, second and four months after Bumetanide treatment showed significantly change KCC2($P\leq0.05$) and NKCC1($P\leq0.001$). Data analyzed by nonparametric repeated measure ANOVA (Friedman’s test) with Dunn's multiple comparisons test; (A) KCC2 expression; (B) NKCC1 expression. Data from each group are presented as box and whisker plot by Tukey method.
Figure 3: KCC2 and NKCC1 protein expression in peripheral blood mononuclear cells was analyzed in healthy control subject’s vs before treatment with Mann-Whitney test, and before vs. after treatment with Wilcoxon matched-pairs signed rank test. (A) KCC2 expression (B) NKCC1 expression. Expression of KCC2 and NKCC1 was normalized to the beta actin (KCC2 and NKCC1/Actin). Each column represents mean ± SD. *P < 0.05; **P < 0.01
Table 1: Demographic and Baseline Clinical Data of the Patients with Neuropathic Pain Following Spinal Cord Injury

1: Time from Injury (TFI): Time Duration after traumatic spinal cord Injury in years.
2: MVA: motor vehicle accidents

<table>
<thead>
<tr>
<th>Age Range (Years)</th>
<th>Subject No/Gender</th>
<th>TFI / Kind of Injury</th>
<th>Symptom Characteristics</th>
<th>Background Medications</th>
<th>Blood Electrolyte Concentration</th>
</tr>
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<tr>
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<tr>
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<td>T6/ MVA²</td>
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<td>Gabapentin, Amitriptyline, Zolpidem, Venlafaxine</td>
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<td>47</td>
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</table>

Frequencies
Table 2: Comparisons of the SF-McGill-Q and m-NRS Responses in patients treated with Bumetanide

Data are presented as mean ± standard deviation (SD) every two month. P values were calculated using mixed model analyzing (MMA). Each items in four parts of SF-McGill-Q including: Continuous, Intermittent, Neuropathic, and Affective is rated based on a 0-10 scale with 0 equal to no pain and 10 equal to the worst pain; SF-McGill-Q, Short-Form McGill Pain Questionnaire; m-NRS, modified Numerical Rating Scale.

<table>
<thead>
<tr>
<th>Number of Items</th>
<th>Item Number</th>
<th>Baseline (N=11)</th>
<th>After 2 month (N=11)</th>
<th>After 4 month (N=9)</th>
<th>MMA</th>
<th>F</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous</td>
<td>6</td>
<td>1, 5, 6, 8, 9, 10</td>
<td>7.11 ± 1.20</td>
<td>6.41 ± 1.06</td>
<td>5.18 ± 1.01</td>
<td>3.286</td>
<td>0.09</td>
</tr>
<tr>
<td>Intermittent</td>
<td>6</td>
<td>2, 3, 4, 11, 16, 18</td>
<td>5.23 ± 1.37</td>
<td>4.56 ± 1.29</td>
<td>4.26 ± 1.22</td>
<td>2.888</td>
<td>0.1</td>
</tr>
<tr>
<td>Neuropathic</td>
<td>6</td>
<td>7, 17, 19, 20, 21, 22</td>
<td>5.68 ± 2.46</td>
<td>4.17 ± 1.74</td>
<td>3.25 ± 1.35</td>
<td>94.237</td>
<td>*** 0.001</td>
</tr>
<tr>
<td>Affective</td>
<td>4</td>
<td>12, 13, 14, 15</td>
<td>7.12 ± 0.81</td>
<td>6.62 ± 0.80</td>
<td>6.3 ± 0.89</td>
<td>2.331</td>
<td>0.14</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>6.2 ± 1.83</td>
<td>5.33 ± 1.68</td>
<td>4.80 ± 1.67</td>
<td>13.138</td>
<td>** 0.002</td>
<td></td>
</tr>
<tr>
<td>NRS Total</td>
<td></td>
<td>7.81 ± 0.87</td>
<td>5.45 ± 1.32</td>
<td>4.22 ± 1.25</td>
<td>3.286</td>
<td>* 0.048</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Comparisons of the eight item-scale of health survey (SF-36) for patients before and after treated with Bumetanide

Data are presented as mean ± standard deviation (SD). P values were calculated using Wilcoxon matched-pairs signed rank test. SF-36 containing 36 questions that includes an 8-scale.

<table>
<thead>
<tr>
<th>Comparisons of the eight item-scale of health survey (SF-36) for patients before and after treated with Bumetanide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Baseline (N=9)</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td><strong>Social functioning</strong></td>
</tr>
<tr>
<td><strong>Physical functioning</strong></td>
</tr>
<tr>
<td><strong>Role – physical</strong></td>
</tr>
<tr>
<td><strong>General health</strong></td>
</tr>
<tr>
<td><strong>Mental health</strong></td>
</tr>
<tr>
<td><strong>Vitality</strong></td>
</tr>
<tr>
<td><strong>Role emotional</strong></td>
</tr>
<tr>
<td><strong>Bodily pain</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

P < 0.05
Table 4: Pearson correlation analysis between SF-36 total score change from baseline with SF-36 subscale scores, NP, SF-McGill-Q total score, and KCC2 that changed significantly in the study

R-values represents the Pearson’s correlation coefficient. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

<table>
<thead>
<tr>
<th></th>
<th>NP</th>
<th>SF-McGill-Q Total</th>
<th>KCC2</th>
<th>NRS</th>
<th>Mental Health</th>
<th>Role Emotional</th>
<th>Bodily Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-36 Total</td>
<td>R</td>
<td>0.186</td>
<td>0.146</td>
<td>-0.042</td>
<td>0.681*</td>
<td>0.725*</td>
<td>0.419</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.583</td>
<td>0.669</td>
<td>0.903</td>
<td>0.021</td>
<td>0.012</td>
<td>0.199</td>
</tr>
</tbody>
</table>
